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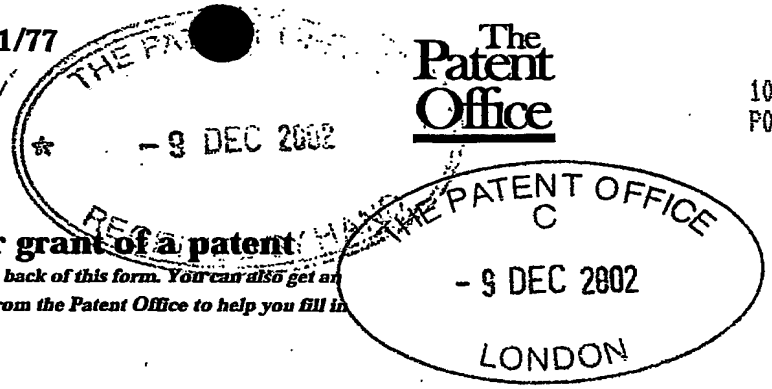

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1/77

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Cardiff Road
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REP06604GB

2. Patent application number
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0228724.1

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ProMetic BioSciences Ltd.
Freeport
Ballasalla
Isle of Man
IM9 2AO

Patents ADP number *(if you know it)*

8030546001

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

MULTIDIMENSIONAL LIBRARIES

5. Name of your agent *(if you have one)*

Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)*

745002

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

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Claim(s)	4
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Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	NO

11. For the applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature Gill Jennings & Every Date 9 December 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

R E Perry
020 7377 1377

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MULTIDIMENSIONAL LIBRARIES

Field of the Invention

This invention relates to multi-dimensional affinity ligand libraries and their preparation, and also to the attachment of ligands to matrices. The invention further relates to the use of the ligands in the purification of natural, recombinant or transgenic proteinaceous materials. The ligands may be also used in medical devices and as a therapeutic drug.

Background of the Invention

The principle of affinity chromatography is based on the phenomenon of molecular recognition. A ligand immobilised on a matrix is able to form a specific, reversible interaction with a target molecule from a mixture of other molecules. The nature of the interaction may be hydrogen bonding, electrostatic forces, stacking as a result of favourable geometry or any other aspect that encourages the host-target relationship. Once bound to the target, the ligand-target interaction should be sufficiently strong to allow the removal of the other contaminant molecules from a mixture while keeping the ligand-target complex intact. However, the binding must be sufficiently weak such that an induced change in chromatographic conditions causes disruption to the interaction, thus releasing the target molecule in its now purified form. The immobilised ligand, now devoid of protein, can be used again for the next purification. Desirable properties of an affinity ligand include chemical and thermal stability, and high selectivity.

The ligand itself may be designed specifically around a particular target molecule as a result of information gleaned about the latter through X-ray crystallography or it may be generated combinatorially in a library of ligands.

The combinatorial approach affords a large number of ligands. A combinatorial library of ligands may consist of only hydrophilic, hydrophobic, charged, neutral or a mixture of chemical moieties. The latter are usually commercially available compounds (amino acids, carboxylic acids, amines etc) or they may be synthesised *in situ* for subsequent attachment to a matrix.

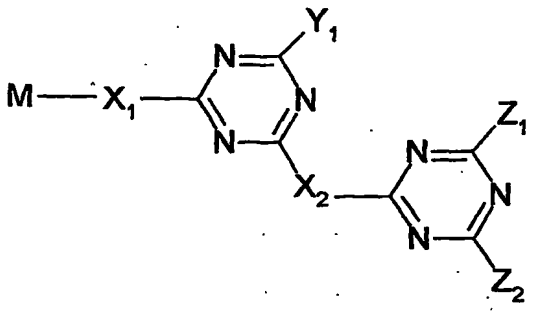
Cyanuric chloride, or 2,4,6-trichlorotriazine (henceforth referred to simply as triazine), is a symmetrical molecule. 2,4,6-trisubstituted triazines can readily

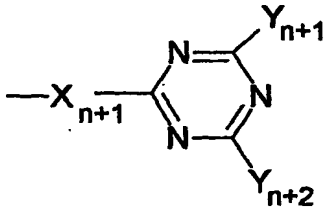
be elaborated to give various compounds, having a defined nucleus. Triazine derivatives are useful as adsorbents and for other purposes; see WO-A-97/10887 and WO-A-00/67900.

Ligand libraries may be built up on a hydroxylic support such as cross-linked agarose. To date, all triazine-based ligand libraries built up on agarose have constituted singly-substituted triazine components; see WO-A-97/10887 or macrocyclic rings incorporating triazine groups; see PCT/GB00/04725.

Summary of the Invention

According to the present invention, a novel compound has the following generic formula I



Where each Z = 

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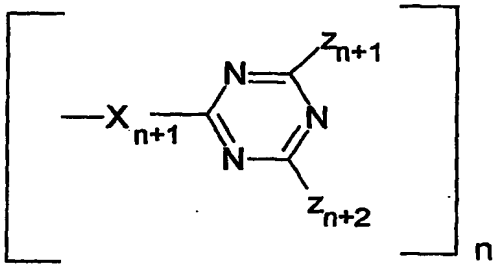

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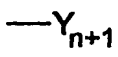


wherein:

each X is a monovalent or multivalent aminyl group that acts as a spacer arm;

each Y is an aminyl group;

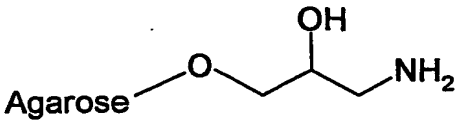
M is a support matrix including any chemical modification required for attachment to an amino spacer group X_1;

Thus by the iterative addition of a spacer (X) followed by a triazine nucleus it is possible to construct a chain of alternating spacer-triazine derivatised ligands. The chlorides of any terminal triazine may be substituted by

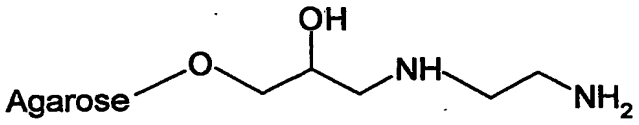
an amine which may itself be multi-functional, optionally with certain functionalities blocked by a protecting group to facilitate oriented attachment. The use of multi-functional amine spacer groups permits the construction of more elaborate ligands leading to branched, antennary, tubular or globular structures.

Description of the Invention

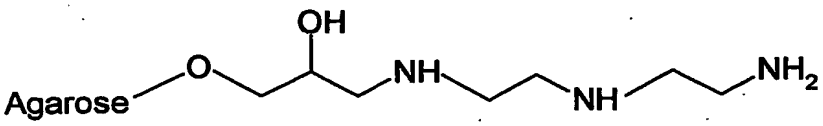
In order to prepare a compound of the invention, a support material such as agarose is subjected to controlled reaction with an activating agent such as epichlorohydrin to introduce reactive groups which facilitate the attachment of an amine spacer X_1. The amount of reactive groups introduced can be measured by suitable assay and expressed in units of μmol/g of settled gel. Excess solvent may be removed by suction or filtration under gravity prior to attachment of an amine spacer group. The activated agarose is first reacted with an amine that provides a spacer (X_1). This may be either monovalent, such as ammonia, divalent, such as 1,2-diaminoethane or trivalent, such as diethylenetriamine or tris(aminoethyl)amine. Assuming a 100% conversion of the reactive group to the amine (*via* nucleophilic addition by the reactant amine), the corresponding aminated resin results. Examples of the product of this first reaction are shown in formulae II, III, IV and V.



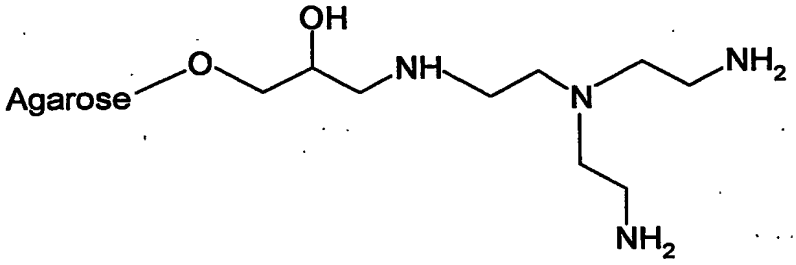
II



III

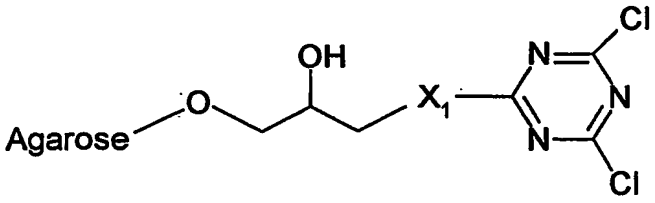


IV



V

The number of amino groups introduced can be determined by any suitable assay, such as TNBS, and may be expressed as μmol amine/g of settled gel. Each of the aliphatic amines introduced at this stage next reacts with cyanuric chloride to generate a triazine-activated agarose formula VI.



VI

An assay for determining the total chlorine content on the triazine activated resin has been developed and the result is expressed in μmol/g settled gel. At this stage, in order to prepare a 3D library, i.e. a compound containing 2 triazine groups and three Y groups, one of the chlorine atoms is substituted by an amine Y_1. The second chlorine atom is replaced by a second

spacer (X_2) which may be a diamine, triamine or tetramine , of any chain length. Each addition of an amine to the triazine ring results in the elimination of a chloride ion, which is assayed and the results are expressed in µmol/g of settled gel.

If the result (in µmol/g of settled gel) from the activation assay = A; the result from the amine assay assay = B; the result from a chloride assay where one chloride ion has been eliminated = C(i), then assuming 100% conversion of starting material to product:

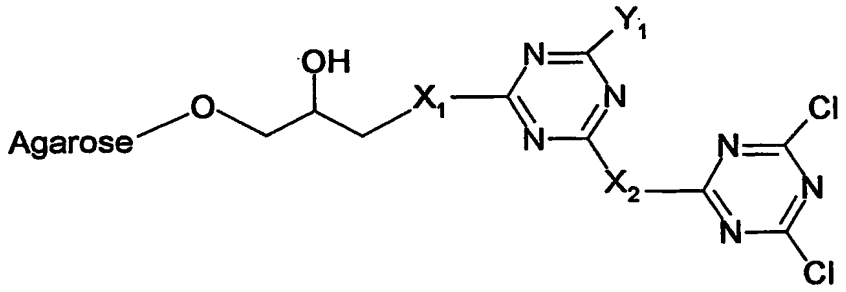
A = B = C(i) (µmol/g of settled gel)

When the second chloride ion, C(ii) has been eliminated, then:

A = B = C(i) = C(ii); and

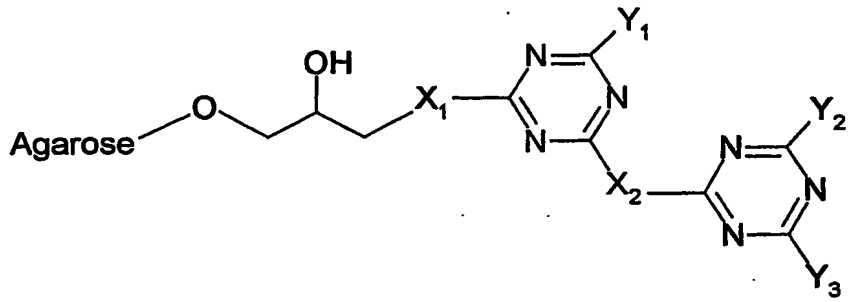
C(i) + (C(ii) = 2A = 2B.

A second triazine substitution step generates a structure of formula VII



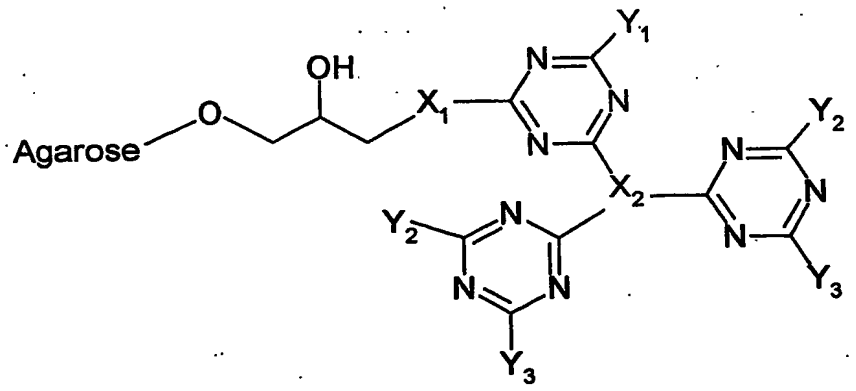
VII

In a 3D library, both chlorine atoms on the second triazine nucleus are substituted sequentially by amines Y_2 and Y_3 to give a product of formula VIII

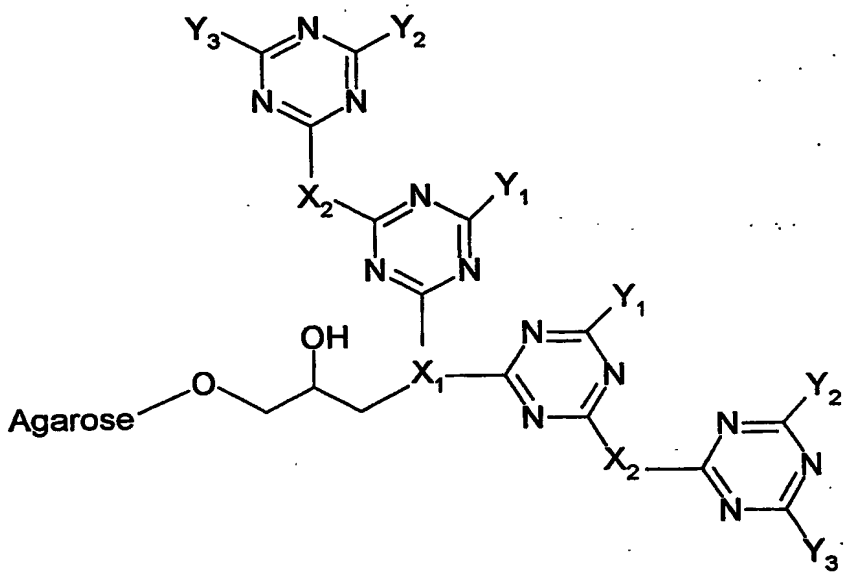


VIII

Examples of other structures built up from these building blocks are shown as formulae IX and X

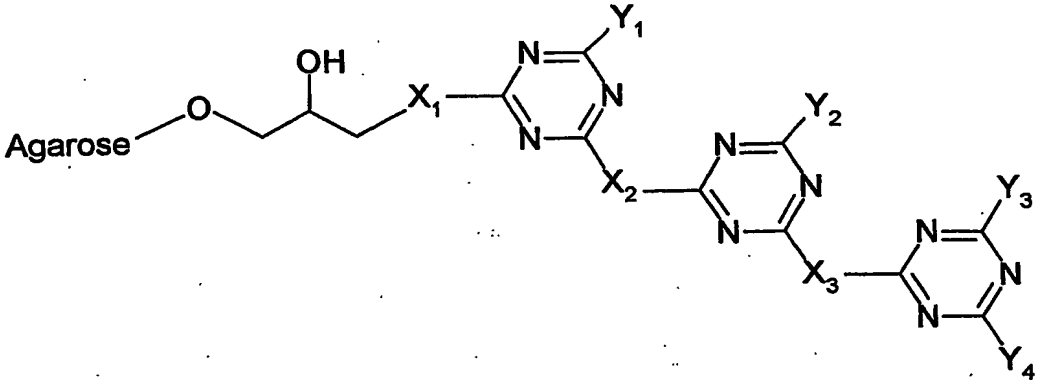


IX

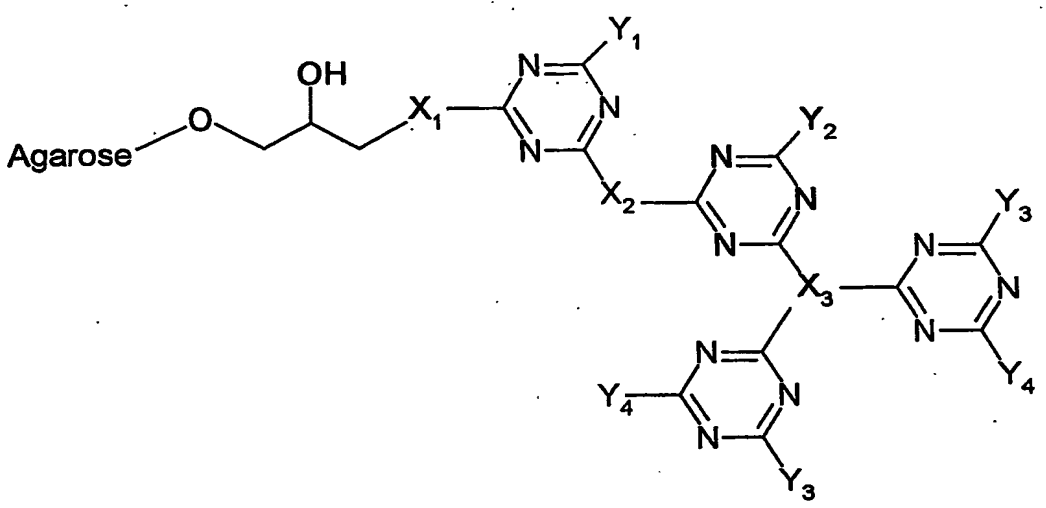


X

Alternating triazine and multivalent spacer groups allows the production of ligands with any number of independently variable amine substitution positions, as exemplified in formulae XI and XII



XI



XII

The following Examples illustrate a 3D library synthesis.

Example 1

This Example illustrates the preparation of epoxide-activated PuraBead resin. PuraBead is modified agarose with increased cross-linking to aid durability.

Epoxide activation: A sample of PuraBead 6XL (333 g) was slurried in water (213 mL) and treated with 10M NaOH (26.6 mL). The mixture was stirred *via* an overhead stirrer until a constant temperature of 34°C was reached. Epichlorohydrin (24 mL) was added in two equal portions and the temperature was increased to 40°C. After 1h, the slurry was filtered and washed with water (10 x 350 mL). Epoxide assay on the settled gel was performed. Obtained, epoxide activated resin (ca 330g, 23.3 μmol/g settled gel).

Example 2

This Example illustrates the preparation of aminated gel from an epoxide-activated gel.

Amine activation: The epoxy-activated gel obtained in Example 1 (226 g) was slurried in water (180 mL) and treated with 0.88 ammonia (45 mL). The reaction mixture was left to stir overnight at 40°C. After this period, the gel was filtered and washed with water (10 x 500 mL) to obtain the amine activated resin (ca 220 g). TNBS assay on a portion of the gel was performed (23.2 μmol/g settled gel).

Example 3

This Example illustrates the nucleophilic addition of aminated resin on to triazine to give the dichlorotriazine activated gel.

Triazine activation: The amine-activated gel (200 g) prepared in Example 2 was washed with 1.0 M potassium phosphate buffer (200 mL, pH 7). The settled gel was transferred to a beaker and treated with water (50 mL) and 1.0M potassium phosphate buffer (50 mL, pH 7). The whole mixture was transferred to a 3-neck 1-litre round bottom flask. Vigorous stirring was maintained as acetone (100mL) was added. The flask was cooled to a steady temperature of 0°C. A solution of cyanuric chloride (5 g) in cold acetone (50 mL) was added from the side arm. The reaction was stopped after 1 h and the flask contents were transferred to a sinter funnel. The dichlorotriazine gel was washed with 50% acetone (1 L), water (1 L), 50% acetone (1 L) and water (2 L) to give the triazine activated resin (*ca.* 200 g). A chloride assay to determine the total chloride ion content was performed (46 μmol/g settled gel).

Example 4

This Example illustrates the addition of the first amine to the dichlorotriazine gel.

Substitution of Y_1: Dichlorotriazine gel (200 g) prepared in Example 3 was slurried in water (100 mL) and treated with a solution of β-alanine (0.2M) in water (100 mL) that had been basified with 10M NaOH (4 mL). The reaction vessel was shaken for 1 h at room temperature after which the contents were filtered and washed with 50% DMF (5 x 125 mL) and water (10 x 125 mL).

Example 5

This Example illustrates the displacement at a higher temperature of the second chloride ion from the triazine component.

Second substitution to add the spacer X_2:The Y_1-substituted gel obtained in Example 4 was slurried in water (100 mL) and treated with a solution of ethylenediamine (0.4M) in water (100 mL). The reaction vessel was allowed to shake at 60°C for 2 days. After cooling, the resultant gel was washed with 50% DMF (5 x 125 mL), water (5 x 125 mL), 0.1M HCl (5 x 125 mL), 30%

isopropanol/0.2M NaOH (5 x 125 mL) and water (10 x 125 mL). TNBS assay was performed on a sample of the gel (26.7 μmol/g). A chloride assay on the supernatant was performed (27.2 μmol/g).

Second Triazine Step: The procedure was identical to the first triazine activation step, described above.

Example 6

This Example illustrates the addition of the first amine to the second triazine ring.

Substitution of Y_2: The gel obtained from the second triazine step in Example 5 was weighed into eight bottles (12.5 g in each bottle). The samples were slurried in 50% DMF (6.25 mL). Each bottle was then charged with a solution of the selected amine (0.2M, 6.25 mL). (Amines containing carboxylate moieties or those obtained as hydrochloride salts were basified with the required volume of 10M NaOH to bring the overall pH to approx. 9-10). The samples were shaken at room temperature for 1 h.

Supernatants (100 mL) were removed from each bottle to assess the progress of the reaction through chloride assay. Table 1 below shows the chloride release figures after the first amine substitution.

Table 1

Column Index in final library	Intermediate Amine	Chloride release (μmol/g)
1	β-alanine	15.61
2	3-aminobenzoic acid	17.13
3	4-aminobenzoic acid	9.83
4	L-glutamic acid	17.52
5	DL-valine	13.97
6	4-aminobutyric acid	18.77
7	L-tyrosine	19.73
8	6-amino-n-caproic acid	17.16

The eight intermediate gels were washed with 50% DMF (5 x 12.5 mL) and water (10 x 12.5 mL).

Example 7

This Example illustrates the combinatorial addition of the final amine (Y_3) on the second triazine ring to give a library of 3D ligands.

Second substitution Y_3: This was afforded by a variation on the method adapted from the second spacer arm (X_2) substitution given in Example 5. The syntheses were performed directly in microspin columns. Alternatively, a 96-well block may also be used.

Each of the eight intermediates (4.0 g) obtained in Example 6 was slurried in 0.4% tween-20 (2 mL). A portion of the slurry (0.375 mL) from the first intermediate was dispensed down a row of eight wells (0.375 mL per well). The process was repeated for the second intermediate down the second row of wells and so on until all eight first stage intermediates had been dispensed down each respective row.

A solution of the first final stage amine (Y_3) (0.4M) in 50% DMF was dispensed along the first row (0.125 mL per well) until all eight wells had been charged. The procedure was repeated for the second final stage amine along the second row until all eight rows had been charged with the selected amine. Thus each well in a library block constitutes an individual ligand.

Once the addition of Y_3 was complete, the library block was placed in a hybridisation oven at 60°C for 2 days. Upon removal and cooling (1h) the block was allowed to drain in to a deep well microtitre plate. The filtrate collected was assayed to determine the release of chloride ion, thus determining the extent of the reaction. The library block was washed with 50% DMF (2 x 1 ml), water (2 x 1 mL), 0.1M HCl (2 x 1 ml), water (2 x 1 mL), 0.2M NaOH/30% isopropanol (2 x 1 mL), water (2 x 1 mL) and 20% ethanol (2 x 1 mL).

The results in Table 2 below show the chloride release data obtained after the second substitution (Y_3) as μmol chloride released per g gel.

Table 2

	1	2	3	4	5	6	7	8
4-(2-aminoethyl)morpholine	13.32	16.68	15.75	16.68	16.45	15.07	16.33	12.8
1-(2-aminoethyl)piperidine	14.29	17.40	14.62	15.52	15.75	15.98	16.92	14.29
3-aminobenzyl alcohol	13.53	16.92	12.18	17.64	15.29	16.56	16.80	14.4
Tyramine	11.49	14.29	11.10	14.07	12.80	13.64	11.88	12.39
2-(p-tolyl)ethylamine	13.43	16.80	15.63	18.63	17.16	16.68	15.41	15.75
Benzylamine	13.11	16.68	14.29	16.21	16.21	16.21	15.29	14.29
Tryptamine	16.68	20.95	19.90	21.08	21.35	20.29	26.74	19.39
1,5-diaminoheptane dihydrochloride	148.15	193.12	155.65	211.67	150.27	209.15	249.96	163.71

The resultant library was screened to test for binding activity/affinity against various target proteins. Table 3 below gives details of a screen performed on human plasma. First the ethanol preservative was washed out of each position in the library by adding 2mL of 25mM sodium phosphate pH7.0 to the top of the gel bed, this was allowed to run through under gravity to displace the 20% ethanol preservative in the gel. Human plasma was diluted 1:20 (v/v) in phosphate buffered saline, 2mL added to the top of each gel bed and allowed to run through under gravity. The flow through (FT) that ran off each library component was collected separately. Each gel bed was then washed to remove non-bound protein by the application of 2mL sodium phosphate buffer pH7.0 in a similar manner; the wash fraction (W) that ran off each library component was collected separately. In the first of two elution steps (E1) designed to remove bound protein, 1mL of 10mM sodium phosphate / citric acid pH6.5 was added to the top of each gel bed and allowed to run through under gravity and collected separately. Next the second elution step (E2) was applied, designed to remove protein not released in the first elution step: 1mL of 50mM citric acid was added to the top of each gel bed and allowed to run through under gravity and collected

separately. Finally a sanitisation step (San) was applied, designed to remove all remaining contaminating material on the gel: 1mL of 0.2M sodium hydroxide / 30% isopropanol was added to the top of each gel bed and allowed to run through under gravity and collected separately.

The protein released in each step was assayed in each of the collected elution fractions: FT, W, E1, E2 and San. The results are shown in Table 3. Each fraction collected from the library is presented as a grid arranged in the same way as Table 2. The figures give the protein recovered from each library component in μg.

Table 3

FT	First Amine							
Second Amine	1	2	3	4	5	6	7	8
9	20967	13050	13863	14722	9075	13050	13863	14722
10	17601	8546	7154	17601	18667	13050	12284	10880
11	18667	7587	9639	15630	7587	7587	9075	10240
12	17601	8546	12284	13863	11561	11561	9639	9075
13	15630	8050	9075	13050	8546	11561	9075	11561
14	17601	7154	10880	13050	14722	12284	10240	13050
15	18667	4572	10880	7154	6749	10880	5094	7587
16	18667	12284	8546	13050	14722	12284	15630	12284

W	First Amine							
Second Amine	1	2	3	4	5	6	7	8
9	740	885	809	740	927	846	971	809
10	774	846	809	1071	774	740	885	740
11	846	846	809	846	1071	740	971	774
12	927	927	809	971	971	774	1128	971
13	1019	971	971	971	1019	846	1128	1071
14	971	1071	1071	971	971	1071	1189	885
15	971	1326	1128	1128	1255	971	1403	1071
16	846	885	885	846	809	774	885	675

E1	First Amine							
Second Amine	1	2	3	4	5	6	7	8
9	878	1727	1459	782	782	694	1544	828
10	464	1101	828	537	464	427	1101	574
11	1165	1930	1826	1165	1459	929	1826	1544
12	1727	2407	1826	1459	1727	1459	1930	1727
13	1633	2407	2041	1544	2041	1380	1826	1826
14	1165	2684	1930	1459	1633	1233	1826	1459
15	1930	2988	2684	1633	2407	1727	2041	2041
16	653	2041	1304	694	574	613	1233	694

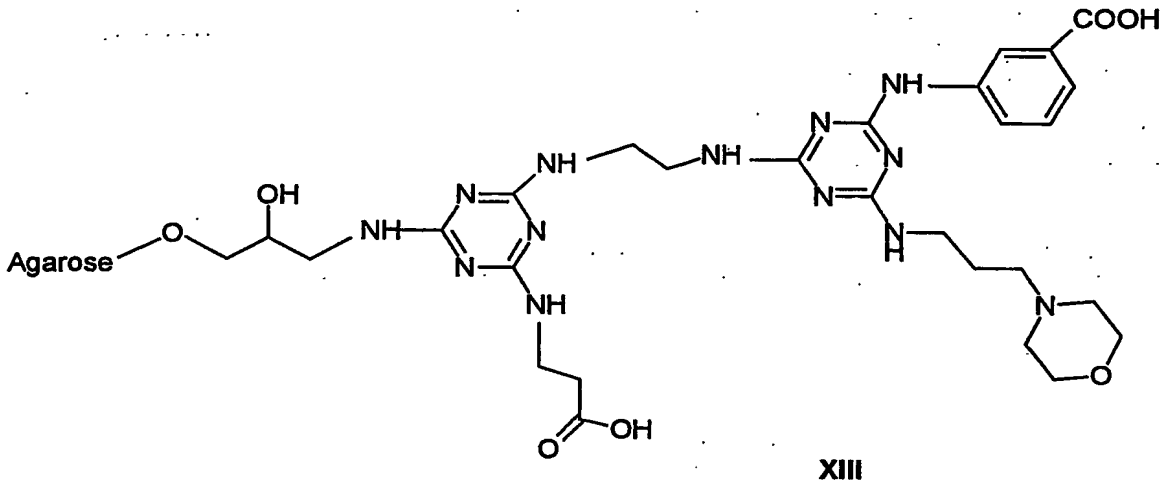
E2	First Amine							
Second Amine	1	2	3	4	5	6	7	8
9	25	178	25	25	0	0	79	25
10	0	25	0	0	0	0	0	25
11	129	904	599	225	270	79	519	225
12	225	1006	810	397	397	129	1614	519
13	313	1793	1531	397	856	225	2213	479
14	438	1531	954	397	558	313	1701	519
15	270	1793	1118	356	680	225	1453	479
16	0	129	25	0	0	0	79	0

San	First Amine							
Second Amine	1	2	3	4	5	6	7	8
9	10	10	0	0	0	0	159	63
10	0	63	0	10	0	0	159	10
11	10	243	159	113	63	10	202	63
12	159	316	281	202	202	63	316	281
13	113	349	316	159	243	113	437	281
14	63	281	202	202	243	202	409	281
15	113	316	243	159	409	63	511	380
16	0	63	0	10	10	0	63	10

Nature of amines (Y_1, Y_2 and Y_3)

The amines selected for the synthesis of 3D libraries may be primary, secondary, aliphatic, aromatic, heterocyclic, aryl, chiral, charged or any combination of these. Reaction conditions may vary with the solubility of the selected amine. Water, 50% DMF and neat DMF are solvents of choice. All amines obtained as hydrochloride salts or containing a carboxylate moeiety are neutralised with the required molar quantity of NaOH before the reaction.

An example of a hydrophilic ligand generated in the 3D library above is represented by XIII.



XIII

wherein

X_1 is derived from ammonia;

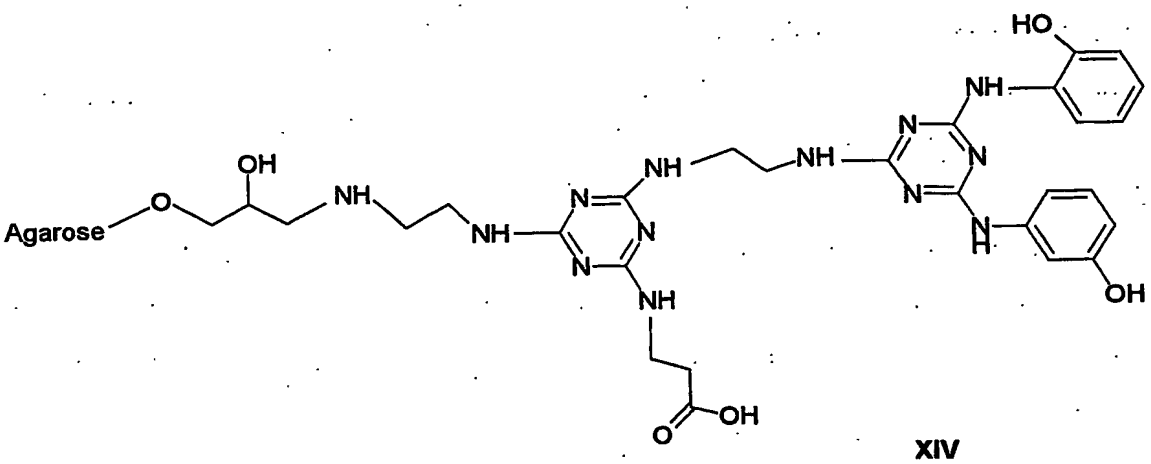
Y_1 is derived from β-alanine;

X_2 is derived from 1,2-diaminoethane;

Y_2 is derived from 3-aminobenzoic acid; and

Y_3 is derived from 1-(2-aminoethyl)morpholine.

Another 3-D ligand represented by structure XIV has been found to be especially useful for the isolation and purification of monoclonal antibodies from cell culture broth and polyclonal antibodies from plasma and plasma fractions.



XIV

wherein

X_1 is derived from 1,2-diaminoethane;

Y_1 is derived from β-alanine;

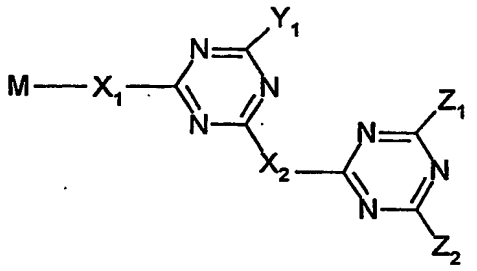
X_2 is derived from 1,2-diaminoethane;

Y_2 is derived from 2-aminophenol; and

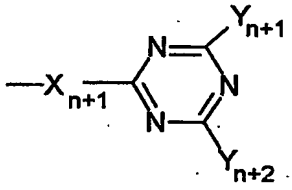
Y_3 is derived from 3-aminophenol.

CLAIMS

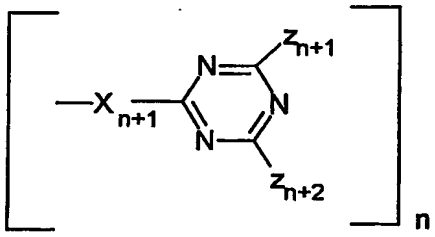
1. A compound of the formula



Where each Z =



or



or

$$—Y_{n+1}$$

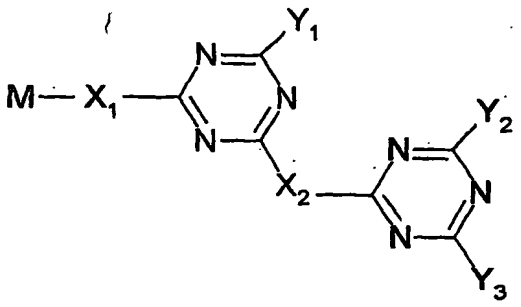
wherein each X is a monovalent or multivalent aminyl group that acts as a spacer arm;

each Y is an aminyl group;

M is a support matrix including any chemical modification required for attachment to an amino spacer group X_1;

or a compound obtainable by elaboration thereof.

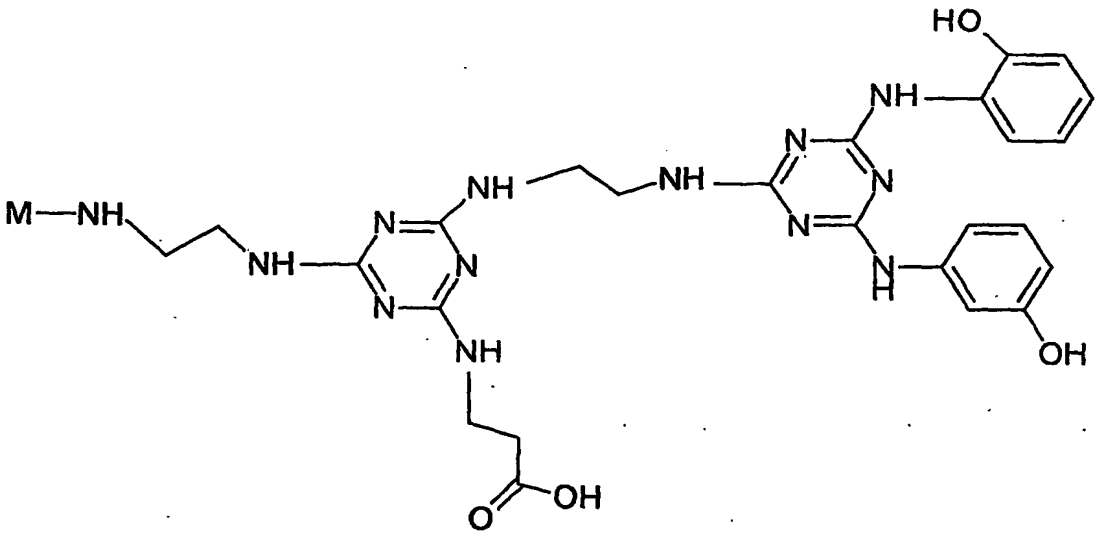
2. A compound of the formula



wherein each X is a monovalent or multivalent aminyl group that acts as a spacer arm and each Y is an aminyl group.

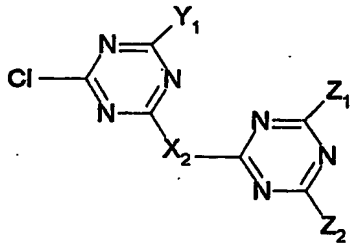
3. Compounds according to claim 2 wherein X_1 and X_2 each independently represent a secondary amino group or a diaminoalkane and Y_1, Y_2 and Y_3 each independently represent an aminyl group selected from optionally substituted aliphatic or aromatic primary amines.

4. A compound of the formula

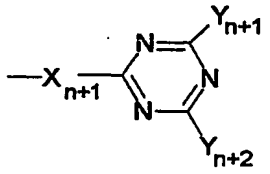


5. A compound according to any one of the preceding claims wherein M represents a support matrix which may be any compound or material, particulate or non-particulate, soluble or insoluble, porous or non-porous which may be used in conjunction with affinity ligands and which provides a convenient means of separating the affinity ligands from solutes in a contacting solution.

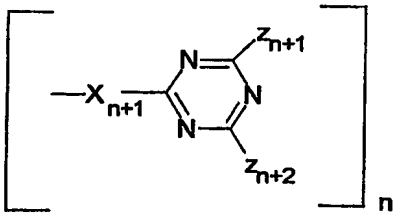
6. A compound of the formula



Where each Z =



or



or

$—Y_{n+1}$

wherein each X is a monovalent or multivalent aminyl group that acts as a spacer arm and each Y is an aminyl group.

7. A method for the synthesis of a compound according to claim 1 by reaction of a compound according to claim 6 with an amine containing support matrix.

8. A method for the synthesis of a compound according to claim 1 wherein a dichlorotriazine moiety is reacted sequentially with an aminyl group Y_1, a spacer group X2, cyanuric chloride, an aminyl group Y2 and an aminyl group Y3.

9. A method for the synthesis of a compound according to claim 1 wherein intermediate structures are synthesised, either singly or in multiples, and then divided into smaller portions for subsequent reaction steps to provide a library of related compounds.

10. The use of a compound according to any one of claims 1 to 6 for the separation, isolation, purification, characterisation, identification, quantification or discovery of peptides and proteins.

11. A process for the separation, purification or discovery of proteinaceous materials comprising carrying out affinity chromatography using a compound according to any one of claims to 6.

12. A process according to claim 11 wherein the proteinaceous material is an immunoglobulin or subclasses, fragments, precursors or derivatives thereof, including fusion proteins, whether derived from natural or recombinant sources.

13. The use of a compound according to any one of claims 1 to 6 for the removal of contaminants, including toxic or pathogenic entities, from preparations of biological or pharmaceutical compounds.